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Induction of MMP-13 expression by soluble human glucocorticoid-induced tumor necrosis factor receptor in fibroblast-like synovial cells[1]

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Summary

Objective: We tested the hypothesis that human glucocorticoid-induced tumor necrosis factor receptor (hGITR/TR11) expressed on the surface of activated $CD4^+$ T cells is responsible for up-regulating the production of matrix metalloproteinase (MMP)-13 by fibroblast-like synoviocytes (FLSs).

Methods: The level of MMP-13 was measured by Western blot and reverse transcriptase polymerase chain reaction (RT-PCR). Expressions of hGITR ligand (hGITRL) on the surface of FLSs and hGITR on the surface of human $CD4^+$ T cells were analyzed by flow cytometry and RT-PCR. Neutralizing antibodies (Abs) were used to block hGITRL and hGITR on the surface of FLSs and human $CD4^+$ T cells, respectively. Human $CD4^+$ T cells were cocultured with FLSs to facilitate interaction between hGITR on $CD4^+$ T cells and hGITRL on FLSs.

Results: Soluble hGITR (shGITR) stimulated FLSs to produce MMP-13, and blockade of hGITRL reduced this effect. Direct contact between activated $CD4^+$ T and FLSs also induced the production of MMP-13, and neutralization of hGITR on activated $CD4^+$ T cells during coculture decreased the amount of MMP-13 produced by FLSs.

Conclusion: shGITR stimulated FLSs to produce MMP-13 via a signal through hGITRL. Direct contact between activated $CD4^+$ T cells and FLSs facilitated hGITR–hGITRL interaction, and resulted in inducing MMP-13. This effect may increase tissue destruction in chronic inflammation such as rheumatoid arthritis (RA).

Key words: Human glucocorticoid-induced tumor necrosis factor receptor (hGITR/TR11), Matrix metalloproteinase (MMP)-13, Fibroblast-like synoviocyte (FLS), Chronic inflammation.

Introduction

The glucocorticoid-induced tumor necrosis factor receptor (GITR), a 66–70 kDa homodimeric glycoprotein, is a member of the tumor necrosis factor receptor (TNFR)-nerve growth factor receptor family induced by dexamethasone in T cells[1]. Human GITR (hGITR/TR11) or human GITR ligand (hGITRL/TL6) was identified by searching for homologues in an expressed sequence tag cDNA database[2,3]. Although the intracellular domains of TNFR members generally have no homology, the intracellular domain of GITR has homology with those of 4-1BB, CD27, and OX40, suggesting that these subfamilies may share signal transduction pathways[3–5]. The expression pattern of GITR in T cells is similar to these other proteins; GITR expression is increased on activated T cells. Shimizu *et al.*[6] have demonstrated that GITR is predominantly expressed on $CD25^+CD4^+$ regulatory T cells in the thymus and its periphery, and that monoclonal antibodies against it abrogate $CD25^+CD4^+$ T cell-mediated suppression *in vitro*. In addition, increased expression of GITR in $CD25^+CD4^+$ T cells has been detected using DNA microarrays[7]. These results suggest that GITR is involved in the control of immunological self-tolerance. In addition to its activity in T cells, soluble GITR (sGITR) activates macrophages and induces nitric oxide synthase[8], cyclooxygenase-2[9], and matrix metalloproteinases (MMPs)[10], probably via GITR/GITR ligand interaction.

Rheumatoid arthritis (RA) is a chronic disabling disease of which progressive destruction of joints and inflammations of the synovial membranes by infiltrated immune cells are important characteristics. Activated fibroblast-like synoviocytes (FLSs) in the lining layer of the synovium contribute to the invasion of articular cartilage and bone. Although normal intimal fibroblasts have not been shown to produce matrix-degrading enzyme, responses to cytokines in culture and *in situ* hybridization studies in diseased tissue suggest that FLSs secrete MMPs more readily than most other

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fibroblasts[11]. FLSs are characterized by elevated expression of adhesion molecules and matrix-degrading enzymes. The latter remove the extra-cellular matrix, providing space for the FLSs to invade. MMPs are a family of zinc-containing endo-proteinases; they include the interstitial collagenases, gelatinases, stromelysins, matrilysin, metalloelastase, and membrane-type MMPs[12]. MMPs are important in tissue remodeling and wound healing, and their expression is regulated at the transcriptional level and also by activation. Effects on MMP expression, either activation, or inhibition by tissue inhibitors of metalloproteinase (TIMPs), may contribute to the progression of diseases such as RA, and osteoarthritis (OA), and to tumor invasion. One of the newest members of the collagenase subfamily, MMP-13, has been shown to degrade collagen types I, II, and III, although it prefers collagen type II which is the primary collagen found in articular cartilage[13]. Transcripts of MMP-13 were originally isolated from breast tumors, and it has been found in articular cartilage and synovial tissues in patients with OA or RA, but not in normal tissue. Cultured FLSs from RA tissue express MMP-13 and can be stimulated with tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)[14].

Activated T cells play a crucial role in the inflammatory process associated with RA. T cells that are actively recruited during inflammation secrete various cytokines that affect the functions of other infiltrating inflammatory cells as well as resident tissue cells such as FLSs. Although many of these observations stem from the examination of individual cytokines, or membrane proteins of T cells, the effect of the expression of hGITR on the surface of infiltrated T cells has not been examined yet. We have tested the hypothesis that hGITR expressed on the surface of activated $CD4^+$ T cells stimulates the production of MMP-13, which presumably contributes to the pathologic remodeling of the extra-cellular matrix, by FLSs. We demonstrate expression of MMP-13 in FLS stimulated with soluble hGITR (shGITR) by Western blot and reverse transcriptase polymerase chain reaction (RT-PCR), and show that hGITRL is expressed on the surface of FLSs. Blockade of hGITRL diminished the level of MMP-13 by inhibiting the effect of shGITR, indicating that up-regulation of MMP-13 involves signaling via the hGITRL in FLSs.

Methods

SYNOVIAL CELL CULTURE

Synovial tissue samples were obtained during operations on patients with OA and RA. All samples were obtained after receiving informed consent. This study was done after approval of the Institutional Ethics Committee of Ulsan University Hospital. Synoviocyte cultures were prepared as described[15] with modifications. Samples were cut into small pieces and dissociated enzymatically with collagenase (0.5 mg/ml, Sigma Chemical Co.) for 16 h at 37°C. The cells obtained were plated on culture dishes in Dulbecco's MEM (DMEM) supplemented with 10% fetal bovine serum (FBS) and allowed to adhere. To eliminate nonadherent cells, the cells were cultured for 48 h, and then washed thoroughly with phosphate-buffered saline (PBS). The adherent synovial cells were grown to confluence, trypsinized and passaged in 150-cm^2 culture dishes. Synovial cells collected at the fourth to eighth passages were used in subsequent experiments. They were a homogenous population of FLSs, and less than 1% reacted with anti-CD45 monoclonal antibody (mAb), or anti-CD11b mAb (data not shown). MH7A cells, an established cell line obtained from RA patients and transfected with SV40 T antigen (Riken Cell Bank, Japan), were used as an FLS cell line. They were grown in RPMI-1640 medium and supplemented with 10% FBS. Cells (10^5 cells/well) were stimulated with the indicated concentrations of shGITR (human TR11-Fc: KOMED, Seoul, Korea).

T CELL CULTURES

Peripheral blood mononuclear cells (PBMCs) were obtained in the form of buffy coats from the Ulsan Red Cross Blood Center and further purified by separation on Histopaque (1.077 g/ml) lymphocyte separation medium. $CD4^+$ T cells were purified using the MACS magnetic separation system according to the manufacturer's instructions (Miltenyi Biotech). Cells were resuspended at 10^8 cells/ml in PBS containing 5% FBS, incubated with antihuman $CD4^+$ mAb conjugated with biotin (B.D. Pharmingen), and collected by incubating with streptavidin-microbeads at 4°C for 15 min. LS columns (Miltenyi Biotech) were used to select the $CD4^+$ T cells, which were routinely $>95\%$ pure by flow cytometry. Human $CD4^+$ T cells were incubated at 10^6 cells/ml in medium with or without phorbol myristate acetate (PMA) (5 ng/ml) and ionomycin (200 ng/ml) or phytohemagglutinin (PHA) (5 μg/ml) for 48 h. For coculture with FLSs, $CD4^+$ T cells were fixed on ice with 1% paraformaldehyde in PBS for 2 h and washed three times with PBS.

IMMUNOBLOT ANALYSIS

Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without a reducing agent and transferred onto nitrocellulose membranes. Some samples were concentrated up to 10-fold by ultrafiltration using membrane units with a molecular weight limit of 10,000 Da (Sigma Chemical Co.). Equal aliquots of the conditioned media, relative to cell number were analyzed for the amount of MMP-13 by Western blotting, using a polyclonal rabbit Ab against human MMP-13 (Chemicon International, Temacula, CA)[16]. The blots were then washed in Tris–Tween buffered saline (TTBS, 20 mM Tris–HCl, pH 7.6 containing 137 mM NaCl and 0.05% (v/v) Tween 20), blocked overnight with 5% (w/v) bovine serum albumin (BSA), and probed with polyclonal antibody (Ab) to MMP-13 in 5% (w/v) BSA dissolved in TTBS. Ab-reactive proteins were detected by enhanced chemiluminescence using horse-radish peroxidase-conjugated secondary anti-rabbit Ab. Antihuman MMP-13 recognizes the latent proenzyme at 60 kDa as well as the active form at 48 kDa. The levels of immunoreactive pro-MMP-13 were quantitated by densitometric scanning of films using an image analyzer.

RNA ISOLATION AND RT-PCR

Expressions of hGITR, hGITRL, MMP-1, MMP-13, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were assessed by RT-PCR. RNA was isolated with TRI reagent (Sigma Chemical Co.). Total RNA was used for cDNA synthesis using the reverse transcriptase in the cDNA synthesis kit (Invitrogen, San Diego, CA). The cDNAs were amplified by PCR for 30 cycles (hGITRL, MMP-1, TIMP-1, and TIMP-2), 35 cycles (hGITR, and MMP-13), and 25 cycles (GAPDH) with the PCR primers listed in Table I. Each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing

Table I
PCR primers used in the study

Gene	Sense primer	Antisense primer
HGITR	5′-GAGTGGGACTGCATGTGTGT-3′	5′-ACAGCGTTGTGGGTCTTGTT-3′
HGITRL	5′-AAGCTGTGGCTCTTTTGCTC-3′	5′-ATACAGCCGCACCTCAAAAG-3′
MMP-1	5′-CACAGCTTTCCTCCACTGCTGCTGC-3′	5′-GGCATGGTCCACATCTGCTCTTGGC-3′
MMP-13	5′-TGGTGGTGATGAAGATGATTTGTCT-3′	5′-AGTTACATCGGACCAAACTTTGAAG-3′
TIMP-1	5′-CCTGGCTTCTGGCATCCTGTT-3′	5′-GGGACCTGTGGAAGTATCCGC-3′
TIMP-2	5′-CAGTGAGAAGGAAGTGGACTC-3′	5′-CATCTGGTACCTGTGGTTCAG-3′
GAPDH	5′-CCACCCATGGCAAATTCCATGGCA-3′	5′-TCTAGACGCCAGGTCAGGTCCACC-3′

Key: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

at 60°C, and 30 s of extension at 72°C. GAPDH was used as internal control. The sizes of the PCR products for hGITR, hGITRL, MMP-1, MMP-13, TIMP-1, TIMP-2, and GAPDH were 250, 281, 396, 376, 280, 300, and 600 bp, respectively.

FLOW CYTOMETRY

Cells (10^6/sample) were incubated in PFS buffer (phosphate based saline, 2.5% FBS, and 0.1% sodium azide) with anti-hGITR Ab (KOMED) (rat immunoglobulin (IgG): isotype control), and anti-hGITRL Ab (KOMED) (rabbit IgG: isotype control) on ice for 30 min to detect hGITR, and hGITRL, respectively. The cells were washed three times in PFS, and incubated on ice for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG, and FITC-conjugated goat anti-rabbit IgG to detect hGITR, and hGITRL, respectively. They were then washed again as above, and flow cytometry was performed using an FACS Calibur (Becton Dickinson).

Results

shGITR STIMULATES SECRETION OF MMP-13

We previously demonstrated that sGITR stimulates murine macrophages via a signal through the GITR ligand[17]. It also induces inflammation in mice[18], and the expression of MMP-9 in murine macrophages[10]. In order to investigate the role of hGITR in chronic inflammations such as RA, we treated FLSs with shGITR and measured the expression of MMP by Western blotting. shGITR-induced MMP-13 in a dose and time-dependent manner, and production of MMP-13 was maximal at 1000 ng/ml shGITR [Fig. 1(A)]. High concentration of shGITR induced the secretion of latent proenzyme at 60 kDa as well as active form at 48 kDa. MMP-13 production was detectible at 15 h after shGITR stimulation and the level increased thereafter [Fig. 1(A)]. Mouse 4-1BB-Fc-treated FLSs, as a negative control, did not produce MMP-13, whereas PMA- or TNF-α-treated FLSs, as positive controls, produced substantial or similar level of MMP-13, respectively, comparing with shGITR-treated one [Fig. 1(A)]. To assess whether shGITR-induced MMP-13 production required *de novo* protein synthesis, we treated FLSs with shGITR in the presence of cycloheximide. No significant retardation of cell growth was observed in cycloheximide-treated cells. As shown in Fig. 1(A), cycloheximide abrogated the stimulatory effect of shGITR on MMP-13 production. This shows that newly synthesized protein is required for, or involved in, the induction of MMP-13 by shGITR. The level of MMP-13 transcripts in the FLSs was measured by RT-PCR. MMP-13 mRNA increased in a time-dependent manner in response to shGITR. It was detectible at 8 h, and reached a maximum at 15–24 h after stimulation [Fig. 1(B)]. Fifteen hours of shGITR stimulation had no effect on the levels of MMP-1, TIMP-1, and TIMP-2 transcripts in FLS, where MMP-1, TIMP-1, and TIMP-2 were constitutively expressed [Fig. 1(C)].

We used a primary RA-FLS and an immortalized rheumatoid FLS cell line, MH7A, to see whether shGITR also increased the expression of MMP-13. A similar pattern of MMP-13 protein secretion was observed in the MH7A cells and primary RA-FLSs [Fig. 2(A) and (B)]. However, in contrast with the OA-FLSs, MMP-13 transcripts were constitutively expressed in MH7A cells and primary RA-FLSs, though at a low level, and started to increase within 6 h of shGITR stimulation [Fig. 2(C)].

shGITR TRANSMITS A SIGNAL THROUGH hGITRL IN FLSs

We tested for the presence of hGITR and hGITRL in primary FLS and MH7A cell lines to examine whether hGITR/hGITRL signaling may play a role in fibroblasts. FACS analysis showed that substantial levels of hGITRL were expressed constitutively on the surface of primary FLS and MH7A cells, whereas no hGITR was detected on them [Fig. 3(A)]. A similar pattern was observed for transcript levels determined by RT-PCR [Fig. 3(B)].

Since FLSs express hGITRL constitutively, shGITR could transmit a signal through it. To determine whether hGITRL stimulates MMP-13 production in FLSs, we added a polyclonal Ab that neutralizes hGITRL to the FLSs in the presence of shGITR. Production of MMP-13 induced by shGITR was inhibited by treatment of this Ab in a dose-dependent manner, but not by rabbit IgG (Fig. 4). These results demonstrate that shGITR stimulates MMP-13 expression in the FLSs via a signal through hGITRL.

MMP-13 PRODUCTION IS MODULATED BY DIRECT CELL CONTACT BETWEEN T LYMPHOCYTES AND FLS VIA hGITR–hGITRL INTERACTION

Contact with stimulated T cells strongly induces MMPs in FLSs, a mechanism that is highly relevant to tissue remodeling in chronic inflammation. The observed effect of shGITR on expression of MMP-13 in FLSs led us to investigate whether induction could be due to direct cell contact between activated T cells and FLSs via hGITR–hGITRL interaction during chronic inflammation. We first determined whether stimulation with PMA/ionomycin (PMA/I) elevated the level of hGITR on the surface of human $CD4^+$ T cells. It has been reported that GITR is mainly expressed on $CD4^+$ T cells, although it is constitutively expressed on both conventional $CD4^+$ and $CD8^+$ T cells[19]. hGITR was found to be constitutively expressed on the surface of

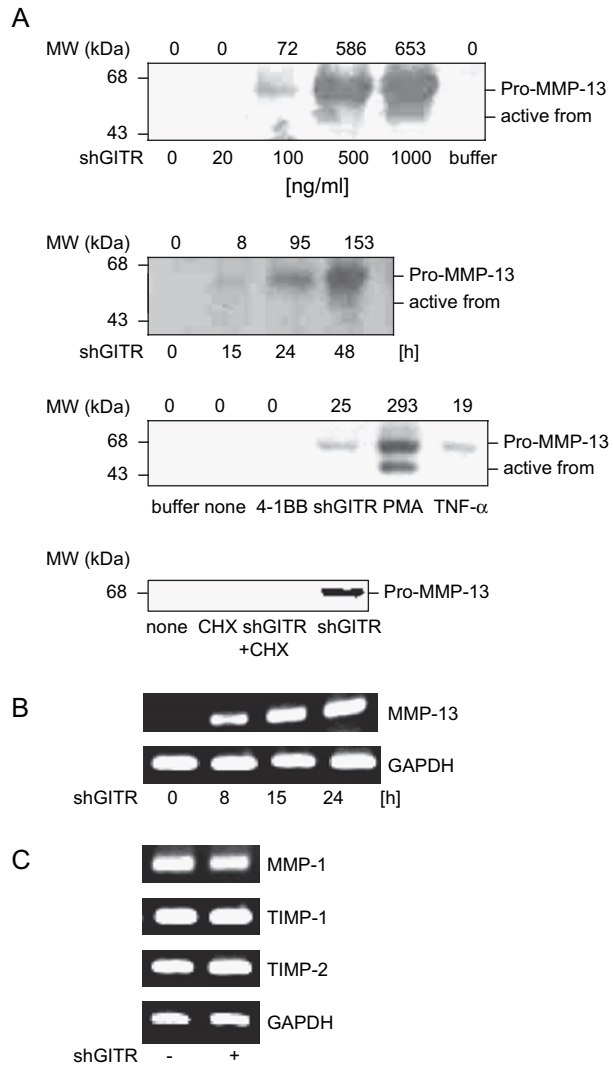

Fig. 1. Effect of shGITR on MMP-13 levels in FLSs. (A) FLSs (10^5 cells/well) were stimulated with shGITR (0, 20, 100, 500, and 1000 ng/ml) for 48 h, or with 500 ng/ml shGITR for 0, 15, 24, and 48 h without FBS. Cells (10^5 cells/well) were incubated for 48 h with murine 4-1BB-Fc (100 ng/ml) and PMA (100 ng/ml) or TNF-α (100 ng/ml) as negative and positive controls, respectively, comparing with shGITR (100 ng/ml). Cells (10^5 cells/well) were incubated with 1 μg/ml cycloheximide in the presence or absence of 200 ng/ml shGITR for 48 h. After incubation, 100 μl samples of the medium were concentrated by ultrafiltration (10-fold), separated on 10% SDS-PAGE gels and analyzed for MMP-13 by Western blotting as described in the Methods section. (B) FLSs (10^6 cells/well) were stimulated with 100 ng/ml of shGITR for 0, 8, 15, and 24 h. Total RNA was extracted and subjected to RT-PCR analysis for MMP-13. (C) Cells incubated with shGITR for 0 and 15 h were analyzed for MMP-1, TIMP-1, and TIMP-2. Similar results were obtained in three independent experiments. Figures above the signals represent the arbitrary densities of pro-MMP-13 expression.

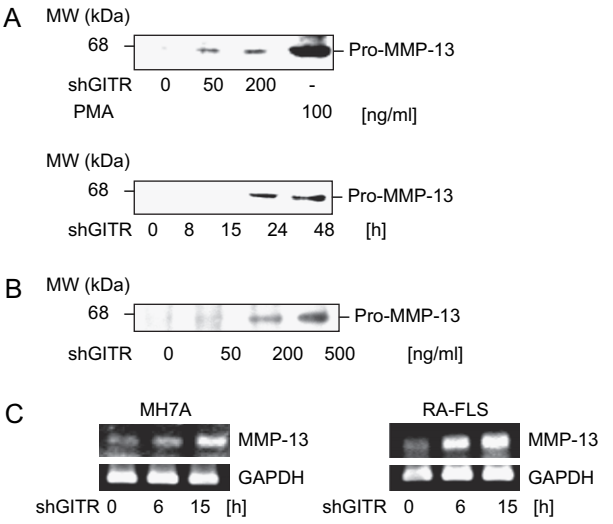

Fig. 2. Effects of shGITR on MMP-13 levels in MH7A and RA-FLS. (A) MH7A cells (10^5 cells/well) were stimulated with shGITR (0, 50, and 200 ng/ml) or PMA (100 ng/ml) for 48 h without FBS. Cells (10^5 cells/well) were stimulated with 100 ng/ml shGITR for 0, 8, 15, 24, and 48 h without FBS. (B) RA-FLS cells (5×10^4 cells/well) were stimulated with shGITR (0, 50, 200, and 500 ng/ml) for 48 h without FBS. After incubation, medium samples of 100 μl were concentrated by ultrafiltration (10-fold), separated on 10% SDS-PAGE gels and analyzed for MMP-13 by Western blotting as described in the Methods section. (C) Cells (10^6 cells/well) were stimulated with 100 ng/ml of shGITR for 0, 6, and 15 h. Total RNA was extracted and subjected to RT-PCR analysis for MMP-13. Similar results were obtained in three independent experiments.

human $CD4^+$ T cells, and expression increased substantially after 2 days of stimulation with PMA/I [Fig. 5(A)]. The level of hGITR transcripts also increased after stimulation [Fig. 5(B)]. Next, we investigated the effect of direct cell–cell interaction on the production of MMP-13. Coculture of FLSs with paraformaldehyde-fixed human $CD4^+$ T cells resulted in MMP-13 production, whereas none was found in the absence of T cells. Coculture of FLSs with both of fixed PMA/I-activated and unstimulated (US) $CD4^+$ T cells produced MMP-13, and higher level was observed in FLS cocultured with stimulated $CD4^+$ T cells [Fig. 6(A)]. There was no difference between $CD4^+$ T cells stimulated by PMA/I and those by PHA [Fig. 6(B)]. In order to identify the molecules present on activated $CD4^+$ T cells that were likely to be responsible for the induction of MMP-13, we used a neutralizing Ab against hGITR. As shown in Fig. 6(C), blockade of hGITR on the activated $CD4^+$ T cells reduced the level of MMP-13, although it did not completely abolish it, comparing with the treatment of rat IgG.

Discussion

We have shown that shGITR activates FLS to secrete MMP-13. Up-regulation of MMP-13 production was also confirmed with a primary FLS and an immortalized FLS cell line, MH7A, from RA patients. Induction of MMP-13 by shGITR was enhanced at the level of transcription. Micro-satellite marker analysis, using sib-pair linkage, has implicated non-HLA-linked loci in human chromosome 1p36 in RA[20]. This locus contains the genes for 4-1BB, CD30, DR3, and CD40, and the gene for hGITR is present in human chromosome 1p36.3[21], although there has been no evidence of an association of hGITR and hGITR polymorphisms with RA. Although sGITR has not been detected in any inflammatory disease or RA patient, it could be involved in inflammatory processes, since sGITR activates murine macrophages to generate nitric oxide, prostaglandin E_2 (PGE_2), and MMP-9[8–10]. A soluble form of TNFR or of a TNF family protein has been detected in

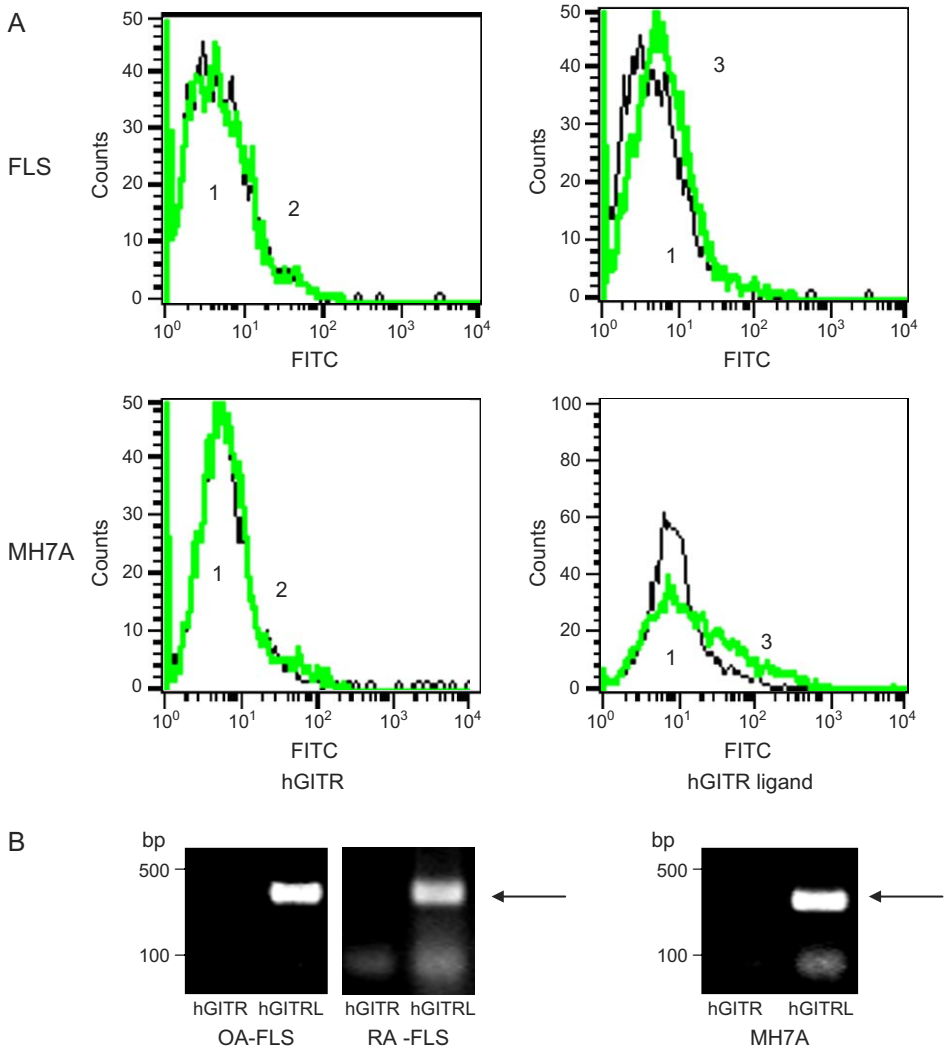

Fig. 3. Expression of hGITR and hGITRL on FLS and MH7A cells. (A) FLS or MH7A cells (10^6 cells/well) were prepared as described in the Methods section and stained with anti-hGITR Ab (2), or anti-hGITRL Ab (3) to detect hGITR [rat IgG: isotype control (1)] or hGITRL [rabbit IgG: isotype control (1)], respectively. The cells were further incubated with FITC-conjugated goat anti-rat IgG or FITC-conjugated goat anti-rabbit IgG for detection of hGITR and hGITRL, respectively, and analyzed by flow cytometry. (B) Total RNA was extracted from OA-FLS, RA-FLS, and MH7A cells (10^6 cells/well) and subjected to RT-PCR. Both cells contain transcript of hGITRL, but not hGITR. Similar results were obtained in three independent experiments.

immuno-pathological disease conditions. Soluble forms of surface receptors can be produced either by proteolysis of membrane-bound receptors, or by alternatively spliced transcripts encoding soluble forms of receptors. In the case of GITR, an alternatively spliced transcript encoding the soluble form has been reported[22]. With regard to other TNFR family proteins, an elevated level of soluble CD27 as well as an increase in the number of CD27 negative T cells were found in synovial fluid in RA, suggesting that activated T cells produce a soluble form of CD27[23]. Soluble 4-1BB has also been found in the sera of patients with RA[24].

Some ligands of the TNF superfamily have been shown to transmit signals in both directions through their respective receptors into cells that express the corresponding ligand, although the mechanism for ligand signaling back into cells is unknown. Since in TNFR and ligand families, the receptors as well as the ligands are membrane-bound molecules, a bidirectional transduction of signals is possible. Bidirectional signals through the receptor as well as the ligand have been described for the OX40, CD40, and CD30 receptor/ligand systems. Reverse signaling through CD30 ligand activates neutrophils to produce IL-8 and a rapid oxidative burst[25]. The signal through 4-1BB ligand inhibits osteoclast formation by interfering with the phosphatidylinositol 3-kinase pathway[26]. Transgenic mice expressing OX40 ligand developed experimental autoimmune encephalomyelitis, indicating a pivotal role for OX40 ligand in the pathogenesis of this disease[27]. hGITRL was originally cloned and expressed in endothelial cells[3], whereas the recently cloned GITR ligand was shown to be expressed in murine macrophages and dendritic cells[28]. Since hGITRL was constitutively expressed on the surface of primary FLS and MH7A cell lines, whereas hGITR was not detectible, the response induced by shGITR could be due to a signal through hGITRL. To establish whether shGITR-stimulated MMP-13 production was caused by signal transduction through hGITRL, we examined the effect of shGITR after blocking hGITRL with a polyclonal anti-hGITRL Ab. Neutralization of hGITRL significantly diminished the effect of shGITR, though it did not block it completely, indicating

shGITR	-	+	+	+	+	buffer
Anti-hGITRLAb	-	-	-	1	5	[ug/ml]
rabbit IgG	-	-	+	-	-	

Fig. 4. The effect of neutralizing Ab against hGITRL on MMP-13 production by FLSs. FLS cells were prepared as described in the Methods section, and were incubated in 24-well plates (10^5 cells/well) for 48 h with polyclonal anti-hGITRL Ab (1, 5 μg/ml) or rabbit IgG (1 μg/ml) in the presence of shGITR (200 ng/ml). After incubation, medium sample of 200 μl was concentrated by ultrafiltration (20-fold), incubated with protein G to remove remaining IgG, separated on 10% SDS-PAGE gels, and was analyzed for MMP-13 by Western blotting. Figures above the signals represent the arbitrary densities of pro-MMP-13 expression.

that hGITRL plays a role in the expression of MMP-13 induced by shGITR. We also showed previously that growth inhibition of murine macrophages by sGITR was due to a signal through GITR ligand[17]. T lymphocytes receive an activation signal from GITR, leading to proliferation[19]. FLS express hGITRL and are activated by cross-linking of the hGITRL. A plot of reverse signaling could be that T lymphocytes and FLS mutually activate each other via interaction of receptor on lymphocytes with its ligand on FLS, leading to an enhancement and maintenance of an immune response such as chronic inflammation.

We have not determined a signaling pathway downstream of hGITRL to generate MMP-13. In case of proinflammatory cytokines, they elicit a series of shared phosphorylation events to facilitate transcriptional induction of MMP-13 beyond the particularities of their receptors. The promoter of MMP-13 contains a TATA box at approximately −30 bp, and an AP-1 site at approximately −70 bp[29]. Murine sGITR activated protein kinase Cδ and phospholipase D to generate MMP-9 in macrophages[30]. MMP-13 expression is also regulated through protein kinase C-dependent pathway[31] and protein kinase C is regarded as a potential inducer of AP-1. It is likely that shGITR-stimulated MMP-13 induction could be under the control of AP-1.

Degradation of articular cartilage is a characteristic of RA. Since MMPs are extremely efficient in cleaving cartilage components, they are considered to play a critical role in the pathogenesis of RA. Of the members of the MMP subfamily, MMP-1 and MMP-13 play major roles in this degradation. The level of MMP-13 induced by shGITR was similar to that generated by TNF-α in FLS. TNF-α has been demonstrated to be predominantly involved in the initiation and progression of articular cartilage destruction. The ability of TNF-α is to promote the synthesis and release of matrix-degrading proteases by human chondrocytes and synovial cells. Among these, the collagenase subfamily including MMP-13 has been demonstrated to play a pivotal role in cartilage destruction[13]. MMP-13 is highly overexpressed in chondrocytes and synovial cells[32]. Since we have not determined shGITR-stimulated MMP-13 by human articular chondrocytes, we cannot say whether there is quantitative difference of MMP-13 level between chondrocytes and synovial cells. However, the localization of MMP-13 implicates its specific roles. Synovial production of MMP-13 is important in the pathogenesis of both arthritides (RA and OA), and in the articular cartilage, MMP-13 participates in tissue remodeling[33]. We found that FLSs from OA patient failed to express transcripts of MMP-13, whereas RA-FLS and MH7A cells expressed MMP-13, though at a low level. At the protein level, secretion of MMP-13 was not detected in US RA-FLS and MH7A cells, probably because of its

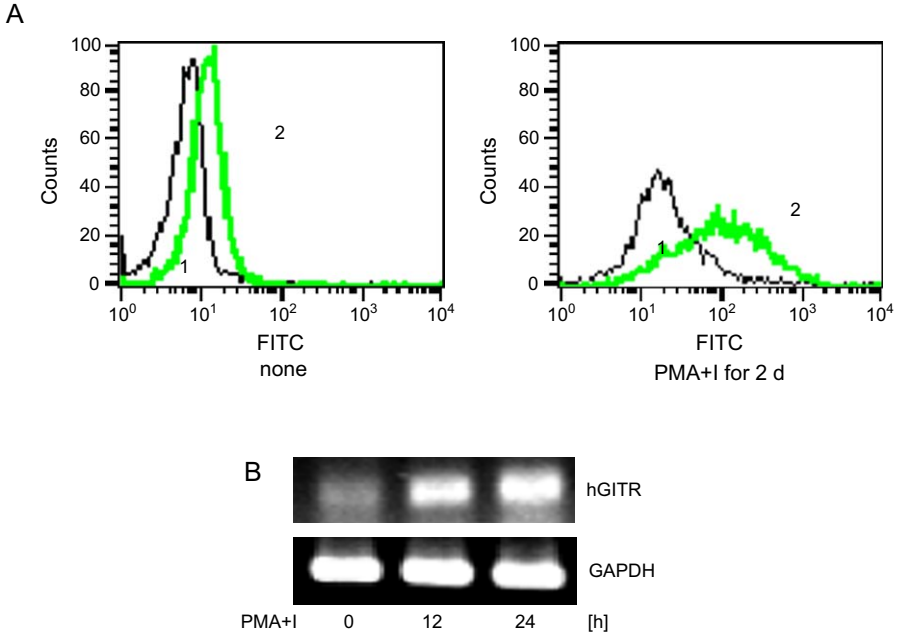

Fig. 5. Expression of hGITR on $CD4^+$ T cells. (A) $CD4^+$ T cells (3×10^6 cells/well) were stimulated with PMA (5 ng/ml) and ionomycin (200 ng/ml) for 2 days and stained with anti-hGITR Ab (2) to detect hGITR [rat IgG: isotype control (1)]. The cells were further incubated with FITC-conjugated goat anti-rat IgG, and analyzed by flow cytometry. (B) Cells (10^7 cells/well) were stimulated with PMA (5 ng/ml) and ionomycin (200 ng/ml) for 0, 12, and 24 h. Total RNA was extracted and subjected to RT-PCR analysis. Similar results were obtained in three independent experiments.

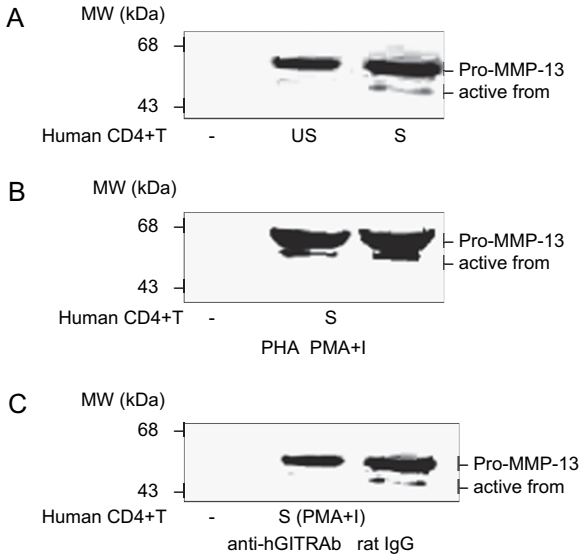

Fig. 6. Effect of direct contact with $CD4^+$ T cells on MMP-13 induction by FLSs. (A) Confluent FLSs (10^5 cells/well) were cultured for 48 h either alone or in the presence of US $CD4^+$ T cells or PMA/I(5/200 ng/ml)-stimulated (S) ones (2×10^6 cells/well). (B) Human $CD4^+$ T cells were stimulated with PMA/I or PHA (5 μg/ml). (C) PMA/I-stimulated $CD4^+$ T cells were pre-incubated with anti-hGITR Ab (10 μg/ml) or rat IgG (10 μg/ml) for 8 h, and incubated with FLSs for 48 h. The $CD4^+$ T cells were fixed as described in the Methods section; before coculture with FLS for 48 h. After incubation, medium samples of 50 μl each were concentrated by ultrafiltration (5-fold), separated on 10% SDS-PAGE gels and analyzed for MMP-13 by Western blotting. Similar results were obtained in three independent experiments.

low level. These results suggest that FLSs could be a source of MMP-13 *in vivo* when inflammatory molecules are present in large quantities due to infiltration of immune cells. The etiologies of RA and OA are different, in that RA is caused by immune dysfunction and chronic inflammation, while OA is the consequence of years of mechanical stress on the articular cartilage. However, a common feature of these two diseases is the proteolytic degradation and ultimate destruction of articular cartilage. Infiltrated immune cells or mechanical insult causes cytokine expression by connective tissue cells such as synovial fibroblast to produce MMPs[14,34]. Although there are profound intrinsic differences between synovial fibroblasts from different sources, we showed that primary OA-FLS and RA-FLS cells and a transformed cell line, MH7A, act in a similar pattern with respect to MMP-13 in response to shGITR.

In many inflammatory diseases where tissue remodeling occurs, mesenchymal cells are in close contact with infiltrated T cells. The mechanism by which T cells contribute to synovial inflammation in RA has not been clarified. However, the recognized HLA associations and the clinical efficacy of T cell-targeted therapies implicate T cells in promoting RA synovitis[35]. Infiltrated T cells may induce inflammation through the release of inflammatory mediators, particularly cytokines, or through pathways dependent on cell contact. T cells can stimulate adjacent cells and modulate the activity of a variety of cell types by cell contact[36]. The role of $CD4^+$ T cells has been demonstrated by the finding that the response to anti-CD4 treatment is correlated with the proportion of synovial $CD4^+$ T cells coated by Ab[37]. It is likely that local positive feedback loops are generated, since FLSs activated by T cells in turn produce cytokines capable of activating T cells. In this work we demonstrated that membrane-associated hGITR is involved in activating FLSs to produce MMP-13 when they interact with $CD4^+$ T cells, since blockade of hGITR decreased the production of MMP-13 by the FLSs. Because neutralization of hGITR on the surface of $CD4^+$ T cells did not completely prevent the production of MMP-13, unknown cell surface factors may be required for $CD4^+$ T cells to stimulate the production of MMP-13 by FLSs. Thus the interaction between hGITR on $CD4^+$ T cells and hGITRL on FLSs could play a substantial role in the production of MMP-13, and this may lead to the extra-cellular matrix degradation characteristic of RA.

In summary we have reported that shGITR stimulates FLSs to produce MMP-13, and the signal involved is transmitted through hGITRL. We also demonstrated an important role in MMP-13 production of contact between hGITR on the surface of $CD4^+$ T cells and hGITRL on FLSs. Direct contact between activated $CD4^+$ T cells and FLSs induces the production of MMP-13 responsible for degradation of type II collagen, a component of the extra-cellular matrix, and this may augment tissue destruction in chronic inflammations such as RA.

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